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Thank you

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eek and *erk*, new members of the *eph* subclass of receptor protein-tyrosine kinases

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We have identified human and rat DNAs encoding two novel members of the *eph* subclass of putative receptor protein-tyrosine kinases. Rat cDNA clones encoding *eek* (*eph*- and *elk*-related kinase) were isolated from a brain cDNA library probed with DNA encoding the kinase region of the insulin receptor-related receptor. The predicted *eek* protein contains all the amino acid residues conserved in the catalytic domains of protein-tyrosine kinases and is most similar to two putative receptor protein-tyrosine kinases of the *eph* subclass, *elk* (69%) and *eph* (57%). Human genomic DNAs encoding part of *eek* (*EEK*) as well as another putative protein-tyrosine kinase most similar to *elk* (90%), *ERK* (*elk*-related kinase), were isolated and partially characterized. The novel identity of these two *eph*-family genes was further supported by Southern blot analyses and localization to human chromosome 1. In Northern blot analysis of rat RNA, DNAs encoding rat *eek* and human *ERK* hybridized to transcripts most abundant in brain and lung, respectively. These two new members of the *eph* subclass of receptor protein-tyrosine kinases, *eek* and *erk*, may therefore have tissue-specific functions distinct from those of other *eph* family members.

Introduction

Protein-tyrosine kinases (PTKs) are structurally and functionally related enzymes intimately involved in signal transduction. Initially discovered as transforming proteins of acutely oncogenic retroviruses (Hunter & Cooper, 1985), altered versions of cellular PTKs have since been implicated in the etiology of certain human malignancies (e.g., Konopka *et al.*, 1984; Martin-Zanca *et al.*, 1986). Under physiological conditions, some PTKs function as receptors for a variety of hormones and growth factors to alter such diverse cellular processes as metabolism, growth and differentiation (Yarden & Ullrich, 1988). Ligand binding to the extracellular region of receptor PTKs somehow activates the cytoplasmic catalytic domain to phosphorylate specific substrates such as the GTPase activating protein (GAP, Kazlauskas *et al.*, 1990), phospholipase Cγ (Meisenhelder *et al.*, 1989) and phosphatidylinositol 3-kinase (Auger *et al.*, 1989).

Receptor PTK subclasses, defined on the basis of structural similarity (Hanks *et al.*, 1988; Yarden & Ullrich, 1988; Ullrich & Schlessinger, 1990), include those of the epidermal growth factor receptor (EGFR), the insulin receptor (IR), and the platelet-derived and fibroblast growth factor receptors (PDGFR, FGFR). Within these subclasses are putative receptor PTKs whose presumptive ligands are unknown (Hanks *et al.*, 1988; Ullrich & Schlessinger, 1990). Insight into the function of these putative receptors will be facilitated if, as expected, family members that exhibit limited divergence play similar roles in cellular physiology (Hanks *et al.*, 1988). It is likely, however, that the elucidation of the functional roles of these PTKs will continue to be outpaced by their rate of discovery.

The *eph* PTK defined a new receptor PTK subclass (Hirai *et al.*, 1987) which on the basis of structural similarity also includes *elk* (Letwin *et al.*, 1988). The *eph* full-length cDNA predicts a transmembrane receptor PTK featuring a single Cys-rich region in the extracellular domain and an uninterrupted PTK domain (Hirai *et al.*, 1987). Two lines of evidence suggest that *eph* may be involved in oncogenesis: *eph* is overexpressed in several human carcinomas (Hirai *et al.*, 1987; Maru *et al.*, 1988); and overexpression of the *eph* gene enabled NIH3T3 cells to form tumors in nude mice and colonies in soft agar (Maru *et al.*, 1990).

We report the isolation and characterization of rat cDNA clones encoding a novel PTK, *eek*, whose predicted amino acid sequence within its kinase domain exhibits extensive similarity with the sequence of receptors belonging to the *eph* subclass of PTKs. Human DNAs encoding *eek* (*EEK*) as well as another novel member of the *eph* subclass, *erk*, have been isolated and used to localize both the *EEK* and *ERK* genes to human chromosome 1. The tissue-specific expression of these two new members of the *eph* subclass of receptor PTKs is distinct from other known members of the *eph* family; *eek* expression is brain-specific and *erk* mRNA is most abundant in lung.

Results and discussion

To identify previously unknown PTKs, we used a DNA probe encoding the kinase region of the insulin receptor-related receptor (IRR, Shier & Watt, 1989) to screen at reduced stringency a rat brain cDNA library. PTKs are abundant in the central nervous system and have been implicated in such brain-specific functions as myelination and neuronal differentiation (Nairn *et al.*, 1985; Edwards *et al.*, 1988). Nucleotide sequence analysis of the entire insert DNA (867 bp) of one cDNA clone which hybridized with the IRR probe, λreek.18, revealed a single open reading frame encoding 289 amino acids. Subsequent screening f another rat brain cDNA library with the insert DNA of λreek.18 identified an overlapping cl ne, λreek.32, that extended the sequence 3′ by 249 nucleotides to a stop codon and a further 1.7 kb to a putative polyadenylation signal. The

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                    ▼D1
reek    RIHIEKIIGSGESGEVCYGRLQVPGQRDVPVAIKALKAGYTERQR    45
relk    FVK..EV..A..F...YK...KL..K.EIY....T.....S.K..    58
heph    WLMVDTV..E..F...YR.T.RL.S.DCKT....T..DTSPGG.W   675
herk                                      ..T..S....KN.    13

                                  ▼D2
heek                              .................T    17
reek    QDFLREAAIMGQFDHPNIIRLEGVVTRGRLAMIVTEYMENGSLDA    90
relk    R...S..S..................KS.PV..I..F....A..S   103
heph    WN.....T.....S..H.LH......KRKPI..I..F...AA...   720
herk    R...S..S.........V.H......KSTPV..I..F.......S    58

        ▼D3
heek    ...                                              20
reek    FLRTHDGQFTILQLVGMLKGVGAGMRYLSDLGYIHRDLAARNILV   135
relk    ...QN.....VI......R.IA...K...EMN.V..........    148
heph    ...ERED.LVPG...A..Q.IAS..N...NHN.V..........    765
herk    ...                                              61

                                  ▼D4
reek    DGRLVCKVSDFGLSRALEDD-PEAAYTTA-GGKIPIRWTAPEAIA   178
relk    NSN............Y.Q..TSDPT..SSL.....V.........   193
heph    NQN.C........T.L.D.--FDGT.E.Q-................   807

                                            ▼D5
reek    FRTFSSASDVWSFGVVMWEVLAYGERPYWNMTNQDVISSVEEGYR   223
relk    Y.K.T.......Y.I.....MSF......D.S.....NAI.QD..   238
heph    H.I.TT........I......SF.DK..GE.S..E.MK.I.D...   852

reek    LPAPMGCPRALHQLMLDCWHKDRAQRPRFSHVVSVLEALVHSPES   268
relk    ..P..D..A..........Q...NS....AEI.NT.DKMIRN.A.   283
heph    ..P.VD..AP.YE..KN..AY...R..H.QKLQAH..Q.LAN.H.   897

reek    LRATATVSRCPA-PAFARSCFDLR--AGGNGNGDLTVGDWLDSIR   310
relk    .KTV..ITAV.SQ.LLD..--IPDFT.FT------..D...SA.K   320
heph    ..TI.NFD--------P.VTLR.PSLS.SD.IPYR..SE..E...   915

reek    MGRYRDHFAAGGYSSLGMVLHMNAQDVRALGITLMGHQKKILGSI   355
relk    .VQ...S.LTA.FT..QL.TQ.TSE.LLRI.V..A.......S..   366
heph    .K..IL..HSA.LDTMEC..ELT.E.LTQM....P....R..C..   981

reek    QTMRSQLSCTQGPRRHL                               372
relk    HS..VQMNQSPSVMA                                 380
heph    .GFKD                                           984
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Figure 1 Predicted amino acid sequences of rat *eek*, human *eek*, and human *erk*, aligned with those of the closely related rat *elk* (Letwin *et al.*, 1988) and human *eph* (Hirai *et al.*, 1987). The rat *eek* sequence numbered from 1 is from λreek.18 with the addition of the 3'-most 83 amino acids from λreek.32. Sequences were aligned using the GAP programme from the University of Wisconsin Genetics Computer Group Sequence Analysis Software Package. Bold letters represent amino acids conserved among kinases (Hanks *et al.*, 1988). Dots replace residues which are identical to the corresponding residues in the rat *eek* sequence. Hyphens represent gaps introduced in sequences to maximize alignment. Triangles demarcate exons D1 to D5 of the *eph* gene (Maru *et al.*, 1988): closed, conserved among *eph* family members where known; open, not conserved. The nucleotide sequences for rat *eek*, human *EEK* and human *ERK* have been submitted to the GenBank™/EMBL Data Bank with accession numbers X59290, X59291, and X59292

composite predicted protein (Figure 1) contains all the amino acid residues conserved in the catalytic domains of PTKs (Hanks *et al.*, 1988) including the potential ATP binding site (Gly^{9}-X-Gly-X-X-Gly^{14} and Lys^{34}). In addition, two sequences (Asp^{127}-Leu-(Ala-Ala-Arg)-Asn^{132} and Pro^{168}-Ile-Arg-Trp-Thr-Ala-Pro-Glu^{175}, Figure 1) specifically conserved in tyrosine rather than serine/threonine kinases as well as a potential phosphorylation site, Tyr^{160}, at a position analogous to the major aut phosphorylation site in $pp60^{v\text{-}src}$ (Smart *et al.*, 1981), are also present. A computer search of sequence databases (EMBL, GenBank and SWISS-PROT, December 1990) revealed that we had identified a novel protein that exhibits striking amino acid similarity in its kinase domain to members of the *eph* subclass of receptor PTKs, *elk* and *eph* (69% and 57% identity, respectively; also see Figure 1). This putative PTK is less similar to PTKs of other receptor as well as non-receptor subclasses: ~32% to 34% identity to representative members of the IR, EGFR, PDGFR, and

FGFR subclasses; and ~40% to 43% with those of the *src*, *abl*, and *fps/fes* subclasses (Figure 1; Hanks *et al.*, 1988; Kornbluth *et al.*, 1988). The similarity between *eek* and the other *eph* family members, *eph* and *elk*, also extends into the carboxy-terminal tail (~43% identity, also see Figure 1), the region thought to exert negative control over receptor PTK signalling function (Ullrich & Schlessinger, 1990). Therefore, we have named this novel putative PTK *eek*, for *eph*- and *elk*-related kinase.

Southern blot analysis of human genomic DNA revealed that a rat *eek* cDNA probe hybridized at reduced stringency to multiple fragments in each digest (Figure 2A), suggesting that this rat *eek* probe could identify several *eek*-related human DNA sequences. At the highest stringency at which any hybridization was observed with the rat *eek* cDNA probe, two human fragments were detected in each digest (Figure 2B). Hybridization with a rat *elk* cDNA probe (Letwin *et al.*, 1988) indicated that one of these fragments encoded the human homologue of rat *elk* (Figure 2C). To confirm that the other hybridizing fragment was the human homologue of rat *eek*, we used rat *eek* cDNA as probe to isolate part of the human *eek* gene (*EEK*) from a human genomic library. The region of human *EEK* homologous to the rat *eek* cDNA probe hybridized selectively to the fragments detected under high stringency by rat *eek* DNA (Figure 2B, D) that had not hybridized with rat *elk* DNA (Figure 2C).

Nucleotide sequence analysis of an ~1 kb fragment of human *EEK* genomic DNA that hybridized with the rat *eek* cDNA probe identified an exon which exhibits high identity with the rat *eek* cDNA (95% amino acid, 92% nucleic acid, Figure 1) and much less similarity with its closest known relative, *elk* (70% amino acid, 71% nucleic acid, Letwin *et al.*, 1988). This *EEK* exon corresponds to amino acid residues 74 to 93 of the rat *eek* cDNA (Figure 1) and to the analogous kinase domain exon D2 of the *eph* gene (Maru *et al.*, 1988). The position of both intron/exon junctions of *EEK* exon D2 (gcccgcccagGC...GGgtgcgt), which are similar to the consensus acceptor and donor splice sites (Breathnach & Chambon, 1981), are conserved between *EEK* and the *eph* gene. Other genes encoding PTKs of the same subclass, such as the *src* (Maru *et al.*, 1988) and the IR (Shier & Watt, 1989) subclasses, have also been reported to exhibit conserved exon/intron organization throughout the entire kinase domain.

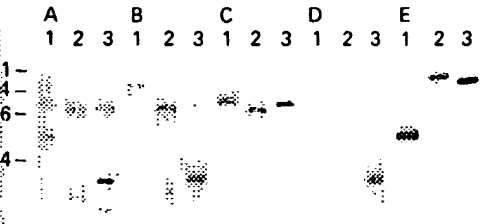

Figure 2 Southern blot analysis of genes encoding members of the *eph* family. Human genomic DNA was digested with EcoRI (lane 1), HindIII (lane 2) or BglII (lane 3). Filters were hybridized with a rat *eek* cDNA probe (reek.D123p, a 246 bp PstI fragment encoding amino acid residues 22 to 103, Figure 1) and washed at 52°C (A) or at 60°C (B); with a rat *elk* cDNA probe (nucleotides 1–238 Letwin *et al.*, 1988; encoding amino acid residues 1–102, see Figure 1) (C), with human *EEK* genomic DNA (hEEK.D2p, an ~1 kb PstI fragment encoding D2) (D), or with human *ERK* genomic DNA (hERK.D12e, an ~400 bp EcoRI fragment encoding part of D1 and all of D2) (E) and washed at 60–62°C

We also isolated another recombinant phage that contained human DNA which hybridized selectively to human genomic fragments (Figure 2E) detected only at reduced stringency by the rat *eek* cDNA probe (Figure 2A). Analysis of the nucleotide sequence of this human isolate revealed that it was most closely related to the *eph* subclass member, *elk* (Letwin *et al.*, 1988); we have named it *erk* for *elk*-related kinase. Over the coding region sequenced, the human *ERK* fragment exhibited high identity (90% amino acid, 81% nucleic acid) with the rat *elk* cDNA (Letwin *et al.*, 1988) and lower identity with its next closest known relative, rat *eek* (74% amino acid, 76% nucleic acid, Figure 1). This human *ERK* genomic fragment (~400 bp from the linker to an internal EcoR1 site) contains a single exon corresponding to part of exon D1 as well as all of exon D2 of the *eph* gene (Maru *et al.*, 1988). The predicted splice junction at the end of exon D2 (CGGgtaggg) is similar to the consensus donor splice site (Breathnach & Chambon, 1981). The lack of an intron between exons D1 and D2 in the *ERK* gene was somewhat unexpected given that this intron is conserved between the genes encoding both *eek* (Figure 1) and *eph* (Maru *et al.*, 1988). Possibly, an intron was lost in *ERK* as a result of reverse transcription of a partially processed pre-mRNA that was re-inserted downstream from a promoter sequence. A similar mechanism of intron loss has been implicated in the rat preproinsulin I gene (Soares *et al.*, 1985).

We have used genomic DNAs from the human *EEK* and *ERK* genes and from 14 human-mouse somatic cell hybrids to localize *EEK* and *ERK* within the human genome. A DNA probe which hybridized specifically with the *EEK* gene, hEEK.D2p, detected a single PstI fragment only in hybrids containing human chromosome 1 (1.0 kb, Figure 3). Similarly, the hERK.D12e probe which hybridized specifically with the *ERK* gene, detected a single PstI human fragment in the same hybrids (4.3 kb, Figure 3). As expected, mouse-specific fragments which hybridized to hEEK.D2p and hERK.D12e were present in all hybrids (3.3 kb and 5.8 kb, respectively, Figure 3). Among all 14 hybrids, chromosome 1 showed 100% concordance with *EEK*

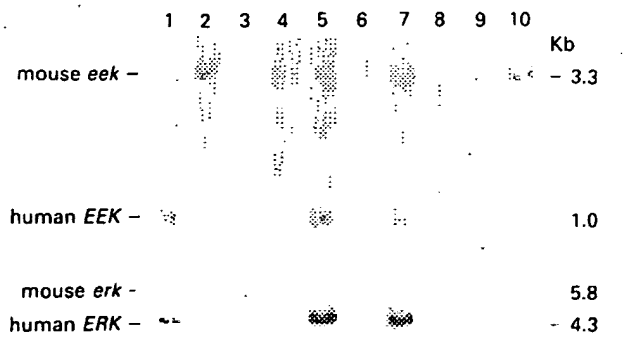

Figure 3 Southern blot analysis of the human *EEK* and *ERK* genes in somatic cell hybrids. Genomic DNA from human (lane 1), mouse (lane 2) and human-mouse hybrid (lanes 3–10) cell lines was digested with PstI and hybridized with human *EEK* DNA (hEEK.D2p, top) or with human *ERK* DNA (hERK.D12e, bottom). Human chromosome 1 and the human DNA fragments hybridizing to *EEK* (1.0 kb) or to *ERK* (4.3 kb) are concordantly present (lanes 3, 5, 7) or absent (lanes 4, 6, 8–10)

Table 1 Segregation of human *EEK* and *ERK* sequences with human chromosomes in somatic cell hybrids

Gene	Chromosome*	Human chromosome†																					
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*X*
+	+	6	2	2	3	2	2	4	3	0	4	2	3	2	4	3	1	1	4	3	4	4	0	5
+	−	0	3	4	3	4	4	2	3	6	2	4	3	4	2	3	5	5	2	3	2	2	6	1
−	+	0	3	2	4	4	2	5	3	0	4	1	4	1	5	2	1	3	2	3	4	4	4	5
−	−	8	5	6	4	4	6	3	5	8	4	6	4	7	3	6	7	5	6	5	3	4	3	0
% discordancy (n = 14)		0	46	43	50	57	43	50	43	43	43	38	50	36	50	36	43	57	29	43	46	43	77	55

* Chromosome scored '+' if present in greater than 10% of metaphases scored
† Chromosomes were not scored if translocations were present in human parental cells

and *ERK* whereas all other chromosomes were excluded by at least 29% discordancy (Table 1).

Our localization of both the *EEK* and *ERK* genes to chromosome 1 demonstrates that these genes map to a chromosomal location distinct from that of the closely related *eph* gene present on human chromosome 7 (Maru *et al.*, 1988). In addition, it raises the possibility that *EEK* and *ERK* may have arisen by duplication of an ancestral gene. A similar gene duplication event has been suggested previously to have given rise to the genes encoding the β type PDGFR and c-*fms*: both have been shown to be on the same chromosome in the human and mouse genomes, and to be tandemly linked in the human genome (Buchberg *et al.*, 1989; Roberts *et al.*, 1988).

The tissue distribution of mRNA which hybridized with DNA encoding the two novel *eph* subclass members was assessed by Northern blot analysis (Figure 4). To ensure detection of only *eek* or *erk* transcripts, we used DNA probes and hybridization conditions which detected only single fragments on Southern blots of rat genomic DNA (data not shown). A rat *eek* cDNA probe containing part of the 3'-untranslated region detected hybridizing transcripts only in rat brain (Figure 4). Even with prolonged exposure, hybridization was not detected in other tissues although multiple larger transcripts were weakly detected in brain (data not shown). In contrast, a human *ERK* genomic probe hybridized to transcripts that were most abundant in lung (Figure 4) and that were also detected on prolonged exposure in placenta, brain, and kidney (data not shown). Hybridization with rat β-actin DNA (Nudel *et al.*, 1983) verified that each lane had approximately the same amount of mRNA and that the mRNA was intact (Figure 4). These tissue distributions of *eek* and *erk* RNAs differ from those of other known members of the *eph* subclass: *elk* mRNA is present in testis as well as in brain (Letwin *et al.*, 1988); *eph* mRNA in kidney, testis, liver as well as in lung (Maru *et al.*, 1988). Also, the sizes of the *eek* and *erk* transcripts (both larger than 28S rRNA, see Figure 4) were larger than those for *eph* and *elk*, large enough to potentially encode an extracellular ligand binding domain as well as the PTK catalytic domain.

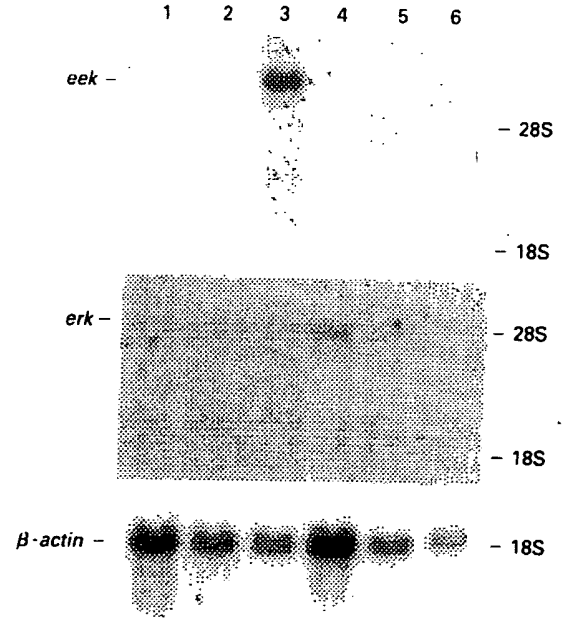

Figure 4 Northern blot analysis of the tissue distribution of *eek* and *erk* transcripts. Poly(A) RNA (2 μg) was extracted from rat small intestine (lane 1), placenta (lane 2), brain (lane 3), lung (lane 4), kidney (lane 5), and testis (lane 6). After gel electrophoresis and transfer, the mRNA was hybridized with a rat *eek* cDNA probe (reek.XE, an ~1.5 kb fragment between ~200 bp 3' to the stop codon and the linker; top), human *ERK* genomic DNA (hERK.D12e, middle), or β-actin DNA (Nudel *et al.*, 1983; bottom); and then washed at 55°C

A comparison of the predicted amino acid sequences of both *eek* and *erk* (Figure 1) suggests that *eek* and *erk* may be new receptor-type PTKs of the *eph* subclass. Primary structures for several members of the *eph* subclass have been predicted to contain potential ligand binding regions (Hirai *et al.*, 1987; Lindberg & Hunter, 1990; Lhotak *et al.*, 1991), although the ligands which bind these putative receptors are currently unknown. Since oncogenic involvement has been implicated for the putative *eph* receptor PTK (Hirai *et al.*, 1987; Maru *et al.*, 1988, 1990), *eek* and *erk* also may play roles in certain types of neoplastic transformation.

Materials and methods

Library screening, cloning and sequencing

A random primed rat brain cDNA library constructed in λgt11 (Auld *et al.*, 1988) was screened with an ~1 kb BamHI fragment encoding part of the kinase domain of guinea pig IRR (residues 1058 to 1194, Shier & Watt, 1989). Insert DNA from one isolate, λreek.18, was then used to screen another rat brain cDNA library (Clontech) as well as a human genomic library in λCharon 4A (Lawn *et al.*, 1978). In each library screen, duplicate nitrocellulose filters were hybridized in 30% formamide at 42°C (Wahl *et al.*, 1979) with DNA probes labelled with [α^{32}P]dCTP (Feinberg & Vogelstein, 1983) and washed in 15 mM sodium chloride, 1.5 mM sodium citrate and 0.1% sodium dodecyl sulfate at 42°C. Insert DNAs from λreek.18, λreek.32, λheek.23 and λherk.7 were subcloned into vectors pEMBL18 or 19 (Allison *et al.*, 1985) and deletions were created by restriction endonuclease digestion. Single-stranded DNA templates were sequenced by the dideoxy

method using the Klenow fragment of DNA polymerase (Sanger *et al.*, 1977), with ambiguities resolved using the modified T7 DNA polymerase and dITP (Tabor & Richardson, 1987; Sequenase, USB).

Southern and Northern blot analysis

Genomic DNAs from human leukocytes, cultured human fibroblasts, mouse fibroblasts or human-mouse somatic cell hybrids (Shier *et al.*, 1990; Watt & Willard, 1990) were digested with restriction endonucleases and size fractionated on 1% agarose gels before transfer to filters (Southern, 1975; Towbin *et al.*, 1979). Poly (A) RNA (2 μg), extracted using guanidine thiocyanate (Chirgwin *et al.*, 1979) and fractionated on an oligo(dT) column, was separated on a 1% formaldehyde-agarose gel (Lehrach *et al.*, 1977) and transferred to nitrocellulose (Thomas, 1980). Rat kidney ribosomal RNA was used as size markers. Filters were hybridized and washed as described above.

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Pasquale et al., Cell Regulation 2(7)523-34, July 1991

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Thank you

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